

Mechanism of Hop-Derived Terpenes Oxidation in Beer

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Terpenes are the main constituents of hops essential oil and contribute to the singular sensory properties of beer. However, terpenes are sensitive to oxidation leading to quality loss during beer aging. Herein, the reactivity of terpenes towards 1-hydroxyethyl radical has been determined employing a competitive kinetic approach using the spin-trap α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitron (4-POBN). The apparent rate constant (k_{app}) for the reaction of terpenes with 1-hydroxyethyl radical ranges from $(3.9 \pm 0.2) \times 10^5$ to $(1.5 \pm 0.2) \times 10^7$ L mol⁻¹ s⁻¹ for β -pinene and terpinolene, respectively. The reaction involves hydrogen atom transfer from the terpene to 1-hydroxyethyl radical rather than electron-transfer and the rate constant is shown to be dependent on the number of allylic and benzylic hydrogen atoms and on the value of the bond dissociation enthalpy for the weakest C–H bond. The results provide a better understanding on the mechanism behind terpene decomposition in beer brewing and aging process and may further contribute to improve the oxidative stability of the herb-flavored beverages.

Keywords: beer, 1-hydroxyethyl radical, terpenes, kinetics, oxidation

Introduction

Hops (*Humulus lupulus* L.) are essential for the brewing process in order to confer the singular sensory properties of beer.^{1,2} Terpenes are the principal constituents of hops essential oil, which make up to 3% (v/m) of the hop cone.³ It is well-known that the composition of hops essential oil depends on the hop genotype, being identified over 200 compounds in its essential oil, some of them have been used for distinguishing among different hop varieties.³⁻⁵ The major terpenes present in hop and beer basically comprise monoterpenes (C₁₀) and sesquiterpenes (C₁₅), which exhibit strong sensory qualities.⁵ The chemical structure of the major brewing terpenes is illustrated in Figure 1, which includes typical terpene hydrocarbons (1-9), terpene epoxide (10) and terpene alcohols (11-16).

Although terpene hydrocarbons like β -myrcene (1), α -humulene (7), β -caryophyllene (8), and β -farnesene (9) have been shown to be the main components of hop oil,^{3,6} the predominant terpenes in finished beer are terpene alcohols due their hydrophilic properties,⁶ especially linalool (14) and geraniol (13), which have been found in appreciable concentrations (ranging from 1 to 906 μ g L⁻¹) depending on the beer type.^{4,7-9} Moreover, several studies have

reported that geraniol (13), nerol (12) and linalool (14) are biotransformed by yeast during the fermentation giving rise to β -citronellol (11) and α -terpineol (15), respectively.^{5,6,10} These yeast biotransformation products are responsible for the typical hoppy aroma of beer.^{5,6,10} However, terpenes are sensitive to oxidation,¹¹⁻¹³ which would result in the loss of beer sensory quality and could yield oxidation products that may display unpleasant organoleptic properties. In view of that, herein we report the reactivity of hop-derived terpenes (1-16) toward 1-hydroxyethyl radical (HER), the predominant radical formed by thermal oxidation during beer brewing process, storage and aging.¹⁴

Experimental

Chemicals and materials

Acetonitrile and ethanol were of HPLC grade and purchased from Panreac (Barcelona, Spain). Ferric chloride tetrahydrate (FeCl₂·4H₂O) and hydrogen peroxide 30% ACS grade were purchased from Merck (Darmstadt, Germany). α -Humulene, (+)- α -pinene, α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitron (4-POBN), α -terpineol, β -caryophyllene, β -citronellol, β -myrcene, (–)- β -pinene, *p*-cymene, 1,4-cineole, *cis*-3,7-dimethyl-2,6-octadien-1-ol (nerol), ferrocene, formic acid, geraniol, limonene,

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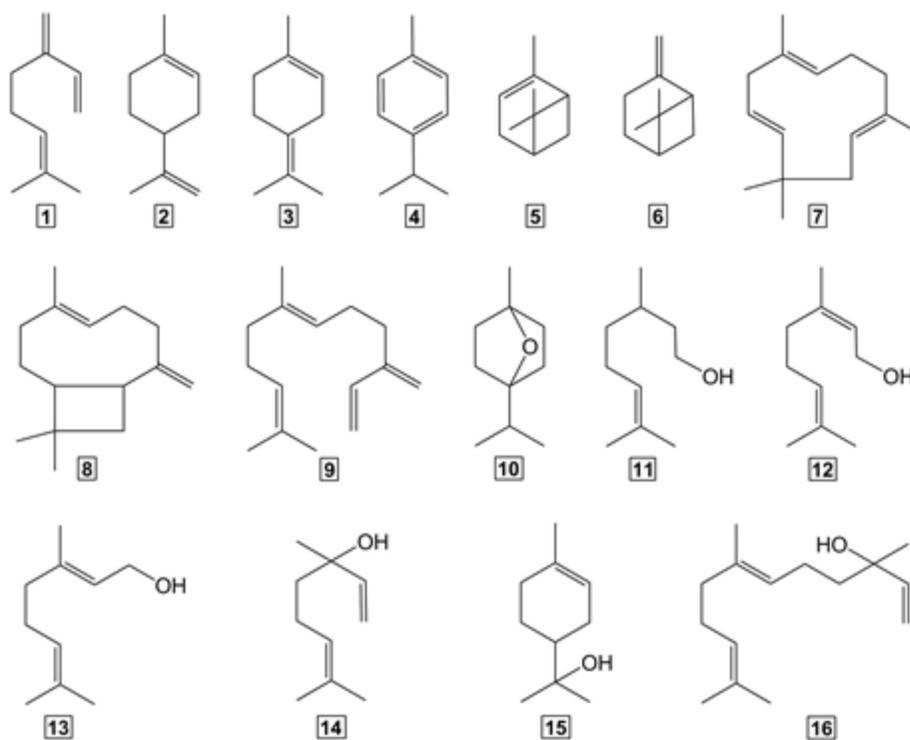


Figure 1. Chemical structures of hop-derived terpenes: (1) β -myrcene; (2) limonene; (3) terpinolene; (4) *p*-cymene; (5) α -pinene; (6) β -pinene; (7) α -humulene; (8) β -caryophyllene; (9) *trans*- β -farnesene; (10) 1,4-cineole; (11) β -citronellol; (12) nerol; (13) geraniol; (14) linalool; (15) α -terpineol; and (16) nerolidol.

linalool, nerolidol, terpinolene, tetrabutylammonium perchlorate, and *trans*- β -farnesene were of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany). Water was purified (18 M Ω cm) by means of a Milli-Q purification system from Millipore (Bedford, Massachusetts, USA). Argon (5.0) was purchased from White Martins (Sertãozinho, São Paulo, Brazil). All solvents used were of HPLC grade and used without further purification.

Formation of HER and competitive kinetics studies using 4-POBN as spin-trap

The formation of HER and the competitive kinetics approach were assayed using 4-POBN as the spin-trap and following the procedure previously reported by de Almeida *et al.*^{15,16} The reactions were conducted in triplicate at 25.0 ± 0.2 °C under argon-saturated atmosphere and started by the addition of 80 μ L of H₂O₂ (58.0×10^{-3} mol L⁻¹) in an ethanolic solution containing 1 mL of 4-POBN (3.2×10^{-3} mol L⁻¹), 60 μ L of FeCl₂·4H₂O aqueous solution at pH 4.5 (2.0×10^{-3} mol L⁻¹), and varying concentrations of terpenes (1-16) in ethanol. After 1 min of incubation, the HER/4-POBN radical adduct was monitored by electron paramagnetic resonance (EPR) spectroscopy. The double integration for calculating the area of the radical

adduct HER/4-POBN EPR signal was measured for each substrate concentration ($n = 3$) and compared with a control experiment ($n = 3$).

Electron paramagnetic resonance (EPR) spectroscopy

The analysis of the paramagnetic spin adduct was performed using a Bruker EMX Plus spectrophotometer (Rheinstetten, Germany), operating at X-band, with magnetic center field of 3472 G, microwave frequency of ca. 9.76 GHz, magnetic field sweep of 50 G, microwave power of 1 mW, and modulation amplitude of 1 G, using a cylindrical ER4103TM cavity and quartz capillary sample cell (ID 0.75 mm, Wilmad Glass, Buena, NJ, US).

Cyclic voltammetry of β -citronellol, limonene, *p*-cymene and α -humulene

Cyclic voltammetry of limonene (2), *p*-cymene (4), α -humulene (7) and β -citronellol (11), were carried out employing a PAR 264A potentiostat and using a boron-doped diamond electrode (8000 ppm) and a platinum wire as a working and auxiliary electrode, respectively. The ferrocene/ferrocenium couple (Fe⁺/Fe) was used as an internal reference, and the measured potential was reported against the normal hydrogen electrode (NHE)

assuming the potential of the Fe⁺/Fe couple equals to E⁰ = +630 mV *versus* NHE in acetonitrile.¹⁷ The compounds (5.0 × 10⁻³ mol L⁻¹ of β-citronellol, 4.1 × 10⁻³ mol L⁻¹ of limonene, 2.5 × 10⁻³ mol L⁻¹ of *p*-cymene, and 1.0 × 10⁻³ mol L⁻¹ of α-humulene) were dissolved in a supporting electrolyte solution of tetrabutylammonium perchlorate (0.1 mol L⁻¹, in acetonitrile) and the solutions were purged with high-purity argon for 15 min before the measurements. The electrochemical cell was thermostated at 25.0 ± 0.2 °C and the scan rate was 100 mV s⁻¹.

Computational methods

Calculations were performed by the Gaussian 09 (G09) program package,¹⁸ edition D.01, employing the DFT method with Becke's three parameter hybrid functional¹⁹ and Lee-Yang-Parr's gradient corrected correlation functional²⁰ (B3LYP) (UB3LYP was used for the radicals species). The 6-31G basis set²¹ was used to the ground-state geometries optimization. Single point energies (SPEs) were carried out by using the 6-311++G(2d, 2p) for the calculation of the bond dissociation enthalpies (BDE). The thermodynamic correction terms (at 298.15 K) were obtained through the calculation of vibrational modes (B3LYP/6-311++G (2d,2p)). The BDEs were calculated according to the formula BDE = H_r + H_H - H, where H_r is the enthalpy of the radical generated by hydrogen atom abstraction, H_H is the enthalpy of the hydrogen atom and H is the enthalpy of the initial molecule. The enthalpy of hydrogen atom used in the BDE calculation was -0.499897 hartree at this level of theory.²²

Results and Discussion

Apparent second-order rate constants

The apparent second-order rate constants for the reaction among selected terpenes and HER were determined by means of a competitive kinetic approach,^{23,24} according to the procedure previously reported in the literature.^{15,16} In this context, the decrease in the HER/4-POBN radical adduct signal intensity resulting from the inhibition of the spin adduct formation due the increasing concentrations of the substrates was probed by EPR spectroscopy, as illustrated in Figure 2 for the presence of β-citronellol (**11**). From the EPR spectra of the HER/4-POBN radical adduct, a triplet of doublets was observed with a nitrogen hyperfine coupling constant of 15.6 G and a hydrogen super hyperfine coupling constant of 2.6 G, which are in agreement with the expected values for the referred spin trapped radical reported in the literature.¹⁴

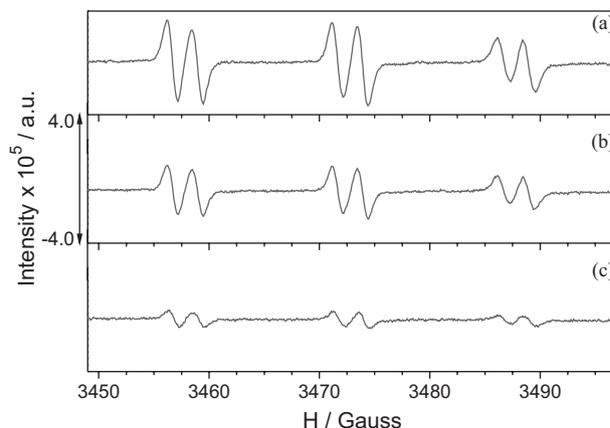


Figure 2. Typical EPR spectra of the HER/4-POBN spin adduct radical recorded for increasing concentrations of β-citronellol (mol L⁻¹): (a) 1.3 × 10⁻³; (b) 3.6 × 10⁻³; and (c) 5.6 × 10⁻³ in the reaction mixture. Reactions were conducted in argon-saturated ethanol solution at 25.0 ± 0.2 °C. The scales in ordinate are the same as showed in the graph.

Thus, by plotting $(F / 1 - F) \times k_2 \times [4\text{-POBN}]$ against the concentrations of the terpenes added to the reaction mixture, as shown in Figure 3 for the β-citronellol (**11**), a linear dependence is observed and the apparent second-order rate constants (k_{app}) may be calculated from the slope of the linear regression fit as established by equation 1.

$$\left(\frac{F}{1 - F} \right) \times k_2 \times [4 - \text{POBN}] = k_{\text{app}} \times [\text{substrate}] \quad (1)$$

where F denotes the percentage of inhibition for the formation of the spin adduct radical, k_2 (3.1 × 10⁷ L mol⁻¹ s⁻¹)²⁵ is the second-order rate constant for the reaction between the spin-trap 4-POBN and HER, and [4-POBN] and [substrate] denote the concentrations of the spin-trap and the selected substrates, respectively.

The apparent second-order rate constants for the scavenging of HER by selected beer terpenes (**1-16**) are

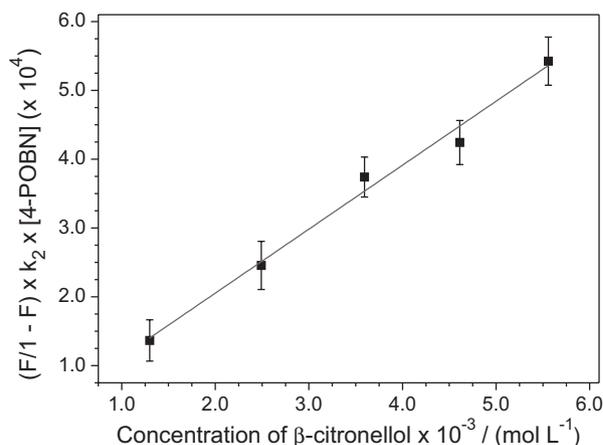


Figure 3. Plot of $(F / 1 - F) \times k_2 \times [4\text{-POBN}]$ versus the concentration of β-citronellol, as obtained by spin-trapping EPR at 25.0 ± 0.2 °C in argon-saturated ethanol solution.

collected in Table 1. As may be observed, even though terpenes exhibit different reactivity toward HER, these substrates proved to be promptly reactive with the major carbon-centered radical formed in beer. According to the orders of magnitude of the obtained apparent second-order rate constants, terpenes may be clustered in four different groups, in which the group 1 is represented by 1,4-cineole (**10**) that did not show any reactivity toward HER, whereas the substrates clustered in groups 2, 3 and 4 exhibit rate constants around 10^5 , 10^6 and 10^7 L mol⁻¹ s⁻¹, respectively.

Table 1. Apparent second-order rate constants (k_{app}) for reaction of selected terpenes with HER. Reactions were conducted at 25.0 ± 0.2 °C in argon-saturated ethanol solution

Group	Substrate	k_{app} / (L mol ⁻¹ s ⁻¹)
1	1,4-Cineole	nr ^a
	β-Pinene	$(3.9 \pm 0.2) \times 10^5$
	α-Pinene	$(4.0 \pm 0.2) \times 10^5$
2	α-Terpineol	$(4.1 \pm 0.2) \times 10^5$
	β-Citronellol	$(4.2 \pm 0.2) \times 10^5$
	Linalool	$(4.2 \pm 0.2) \times 10^5$
	<i>p</i> -Cymene	$(5.4 \pm 0.1) \times 10^5$
	β-Myrcene	$(1.6 \pm 0.3) \times 10^6$
3	Limonene	$(2.1 \pm 0.1) \times 10^6$
	β-Caryophyllene	$(2.3 \pm 0.1) \times 10^6$
	Nerol	$(3.5 \pm 0.2) \times 10^6$
	Geraniol	$(3.6 \pm 0.1) \times 10^6$
	Nerolidol	$(3.7 \pm 0.2) \times 10^6$
4	β-Farnesene	$(5.4 \pm 0.1) \times 10^6$
	α-Humulene	$(1.3 \pm 0.2) \times 10^7$
	Terpinolene	$(1.5 \pm 0.2) \times 10^7$

^anr = not reactive.

The difference in reactivity observed for the reaction of terpenes with HER probably is due to the presence of different reactive sites at the substrate structures. Several studies appoint that reactions involving terpenes and radical species are generally initiated by hydrogen atom transfer (HAT) from allylic or bisallylic C–H group of terpenes to the radical species rather than proceed through an electron transfer (ET) mechanism, which may explain the lack of reactivity of 1,4-cineole (**10**) towards HER.^{11,12}

In order to rule out the ET mechanism for the reaction of terpenes with HER, the oxidation potentials of limonene (**2**), *p*-cymene (**4**), α-humulene (**7**) and β-citronellol (**11**), were determined by cyclic voltammetry, as displayed in Figure 4. Cyclic voltammetry experiments provide the anodic potentials of +2.7, +2.2, +2.6 and +2.7 V for (**2**),

(**4**), (**7**) and (**11**), respectively, relative to NHE, as result of an irreversible one-electron processes. By comparing the oxidation potential for these compounds with the reduction potential of HER (0.98 V relative to NHE),²⁶ we may infer that ET reaction of HER and hop-derived terpenes is thermodynamically uphill, which corroborates with the fact that the investigated reaction is governed by HAT rather than ET.

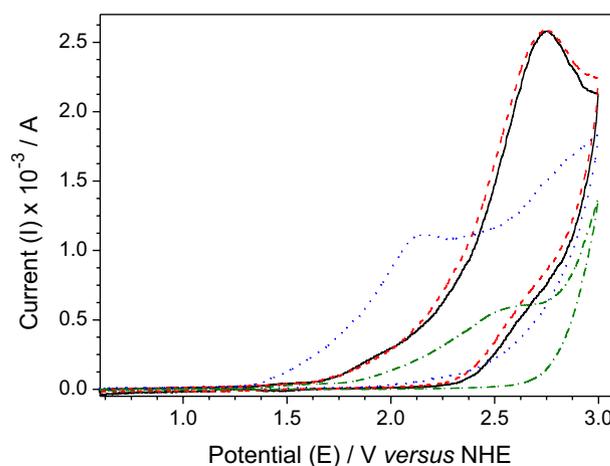


Figure 4. Cyclic voltammograms of (---) β-citronellol (5.0×10^{-3} mol L⁻¹), (—) limonene (4.1×10^{-3} mol L⁻¹), (···) *p*-cymene (2.5×10^{-3} mol L⁻¹), and (-.-) α-humulene (1.0×10^{-3} mol L⁻¹) dissolved in the supporting electrolyte tetrabutylammonium perchlorate (0.1 mol L⁻¹, in acetonitrile), at 25.0 ± 0.2 °C, and scan rate of 100 mV s⁻¹.

Aiming to compare and provide a linear free energy relationship for the apparent rate constants obtained for the reaction between HER and terpenes, BDEs for the relevant allylic, bisallylic and benzylic C–H and O–H bonds were calculated using the density functional theory (DFT) method. From the data obtained by *ab initio* DFT calculations displayed in Figure 5, it may be noticed that allylic, bisallylic and benzylic C–H bonds present lower BDE values than the O–H bond, as calculated for geraniol (**13**), with exception for the allylic bridge C–H bonds as existent in the chemical structures of α- (**5**) and β-pinene (**6**), which shown BDE values higher than 400 kJ mol⁻¹. Thus, is reasonable to infer that the oxidative reaction is initiated by hydrogen abstraction from the allylic, bisallylic and/or benzylic methylenic hydrogen by HER.

The most reactive terpenes were terpinolene (**3**) and α-humulene (**7**), with apparent rate constants of ca. 10^7 L mol⁻¹ s⁻¹. This high reactivity may be accounted mainly by the presence of bisallylic hydrogen atoms on the referred chemical structures. Indeed, the verified BDE values for bisallylic C–H bond (294 kJ mol⁻¹) of terpinolene (**3**) was at least 33 kJ mol⁻¹ lower than the respective values obtained for the allylic C–H bonds of the referred substrate (BDEs range from 327 to 354 kJ mol⁻¹),

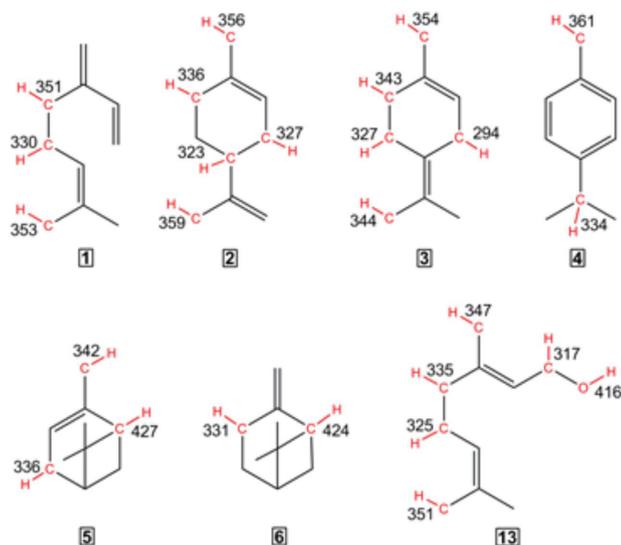


Figure 5. Lowest bond dissociation enthalpies (BDEs, in kJ mol^{-1}) for the allylic, bisallylic and benzylic C–H and O–H bonds of terpenes as calculated by DFT (6-311++G(2d,2p) basis set) method for (1) β -myrcene, (2) limonene, (3) terpinolene, (4) *p*-cymene, (5) α -pinene, (6) β -pinene, (13) geraniol.

which indicates the facility to abstract the bisallylic hydrogen atoms by the radical rather than the allylic and benzylic hydrogen atoms. On the other hand, the higher BDE values obtained for primary carbon of allylic non-bridge C–H bonds (BDEs range from 344 to 359 kJ mol^{-1}) compared to the values for secondary (BDEs range from 317 to 351 kJ mol^{-1}) and tertiary (BDE of 323 kJ mol^{-1}) carbon of allylic non-bridge C–H bonds indicates that the abstraction may occur on the allylic non-bridge hydrogen atom attached to the tertiary and secondary carbons rather than the respect hydrogen atom linked to the primary carbons. Added to this fact, it is well known that tertiary

and secondary radicals are more resonance-stabilized than the respective primary radicals.

Therefore, the thermal oxidation of unsaturated hop-derived terpenes in beer was found to occur preferentially by the abstraction of bisallylic hydrogen atom from the substrates to HER, followed by the abstraction of allylic non-bridge hydrogen atom connected to the tertiary and secondary carbons, leading the respective bisallylic and allylic radical derived from terpenes, as illustrated for limonene (2) and terpinolene (3) in Figure 6. In this context, the main radical species generated during limonene (2) oxidation by HER is the allylic radical formed from the abstraction of the hydrogen atom linked to tertiary and secondary carbons; on the other hand, for the terpinolene (3), the principal primary oxidation products is the bisallylic radical. For the benzylic compounds, here represented by *p*-cymene (4), the C–H BDE value involving the tertiary carbon was expressively lower ($\Delta\text{BDE} = 27 \text{ kJ mol}^{-1}$) than the obtained for the referred primary carbon, suggesting the significant hydrogen atom donor property of benzylic hydrogen atom attached to the tertiary carbon.

Moreover, from the apparent second-order rate constants obtained for the reaction involving terpenes and HER, it may infer that the reactivity of terpenes towards HER has a linear dependence on the number of hydrogen atoms susceptible to abstraction, as displayed in Figure 7. Also, it may be seen in Figure 7 that the terpinolene (3) behaves as an outlier in the tendency, since it presents a bisallylic hydrogen atom rather than an allylic or benzylic hydrogen atom.

The relationship between $\log(k_{\text{app}})$ versus the lowest C–H BDE value for the allylic, bisallylic and benzylic H-atom in the selected substrates shows a satisfactory linear correlation, as may be seen in Figure 8. This free

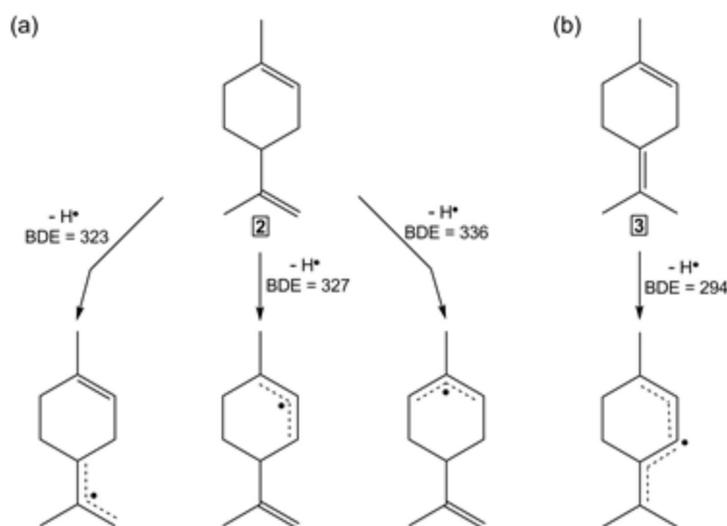


Figure 6. Illustration of the main radicals that may be formed by hydrogen atom abstraction from (2) limonene (a) and (3) terpinolene (b) by HER and the respective bond dissociation enthalpy (BDE) values (in kJ mol^{-1}) for the allylic and bisallylic C–H bonds, respectively.

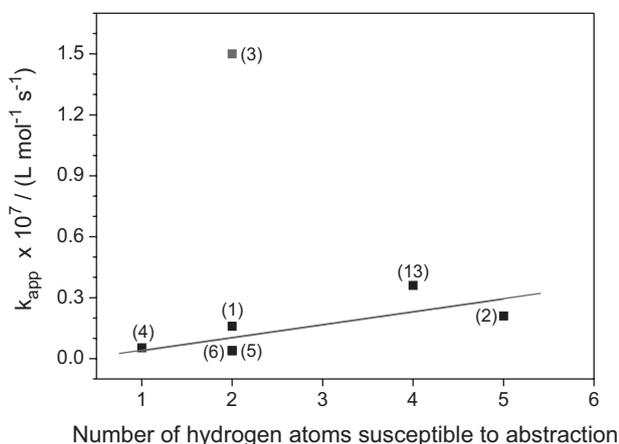


Figure 7. Plot of the apparent second-order rate constants (k_{app}) versus the number of hydrogen atoms of (1) β -myrcene, (2) limonene, (3) terpinolene, (4) *p*-cymene, (5) α -pinene, (6) β -pinene, and (13) geraniol susceptible to abstraction by HER.

energy relationship indicates that as higher as the C–H BDE value is, lower is the apparent second-order rate constant, suggesting that the activation energy for the hydrogen atom transfer increases with the C–H BDE values, as expected.^{27,28} Moreover, this linear relationship indicates that the studied molecules have the same reaction mechanism (HAT) toward HER. From this analysis, the best linear regression fit of the data is described in equation 2, by which the apparent rate constants of the reaction between terpenes and HER can be estimated.

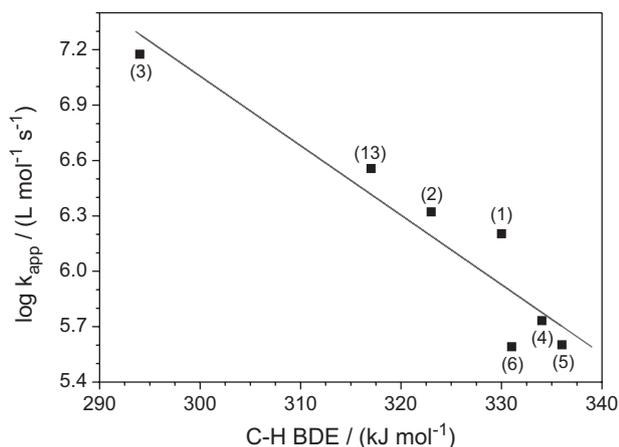


Figure 8. Plot of logarithm of the experimental apparent second-order rate constant ($\log(k_{app})$) for HAT from (1) β -myrcene; (2) limonene; (3) terpinolene; (4) *p*-cymene; (5) α -pinene; (6) β -pinene; and (13) geraniol to HER versus the minimum of BDE values determined for the weakest C–H bonds.

$$\log(k_{app}) = -0.038 \times (\text{C–H BDE}) + 18.3 \quad (2)$$

The reactivity of some terpenes present in herb-flavored beers like 1,8-cineole, menthol, limonene, carveol and carvone was investigated toward triplet-excited-state of

riboflavin, a well-known photosensitizer present in beer, and no reaction with the triplet-excited flavin was observed.²⁹ This suggests that the main degradation mechanism of terpenes in brewing process and beer aging is associated with the thermal oxidation, which involves the formation of HER. Concerning the thermal oxidative process in beer, the reactivity of beer hop-derived bitter acids, prenylflavonoids, phenolic compounds, thiol-containing compounds towards HER was previously reported, in which beer bitter acids, thiols and prenylated flavonoids showed similar apparent rate constants, with magnitude orders of 10^8 and $10^9 \text{ L mol}^{-1} \text{ s}^{-1}$.^{15,16,30,31} On the other hand, the second-order rate constants verified for the reaction between phenolic compounds and HER are around $10^4 \text{ L mol}^{-1} \text{ s}^{-1}$.³² The average content of prenylflavonoids and thiols in beer is around 4-³³ and 20-fold³⁴ higher than the content of hop-derived terpenes, respectively, whereas bitter acids and phenols are present in concentrations around 127-²⁹ and 1000-fold²⁹ higher than terpenes, respectively. Thus, considering the rate constants obtained for the reaction involving HER and these substrates and their content compounds in beer, we may infer that phenolic compounds and hop-derived terpenes may scavenge only a minor amount of HER and are not expected to display a relevant role in the antioxidant properties of beer.

Conclusions

Hop-derived terpenes were shown to be reactive toward HER, being the reaction preferentially governed by the abstraction of allylic non-bridge, bisallylic and benzylic hydrogen atoms from terpenes to HER. The reactivity of terpenes with HER showed to be dependent on the number of allylic non-bridge and benzylic hydrogen atoms susceptible to abstraction on the substrates and on the C–H BDE values respect to allylic, bisallylic and benzylic hydrogen atoms, in which as higher as the number of oxidizable methylenic hydrogen atoms present on the substrate structures and as lower as the BDE values is, higher is the apparent second-order rate constants of the investigated reaction. Although the thermal oxidation is in a lower extension to terpenes compared with that verified for beer bitter acids, prenylflavonoids and thiols, the results of the present study may contribute to better understanding of the mechanism behind the decomposition of terpenes in beer brewing process and aging, which may contribute to the oxidative stability of the herb-flavored beverage.

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